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# *Mycobacterium avium-Mycobacterium intracellulare* Complex-Induced Suppression of T-Cell Proliferation In Vitro by Regulation of Monocyte Accessory Cell Activity

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**Heat-killed whole *Mycobacterium avium-Mycobacterium intracellulare* complex (MAC) and its lipid component impaired the capacity of human peripheral blood mononuclear cells to proliferate in vitro in response to concanavalin A (ConA), purified protein derivative of tuberculin (PPD), and to a lesser degree, phytohemagglutinin stimulation. Inhibition by MAC was not contingent upon prior exposure of the donor to MAC or other mycobacteria and occurred with lymphocytes from tuberculin-negative as well as -positive subjects. The suppression was not due to the toxicity of MAC. The suppression by MAC was not blocked by indomethacin. Adherent cell depletion and cell mixing experiments with T cells indicated that monocytes and not T cells were a major contributor to the immunosuppression observed. However, neither interleukin-1 production nor the expression of HLA-DR (Ia antigen) by monocytes was suppressed by MAC treatment. On the other hand, treatment of monocytes with MAC or MAC-derived lipid resulted in significant decreases in CD11b, a member of the leukocyte function-associated molecule-1 and LeuM3 (CD14) molecule. Anti-CD18 (β-chain of the leukocyte function-associated molecule-1 family) monoclonal antibody had suppressive effects on ConA- and PPD- but not phytohemagglutinin-induced in vitro lymphocyte blastogenesis. We suggest that MAC and MAC-derived lipid suppress the ConA- and PPD-induced T-cell proliferations by blocking the expression of accessory molecules on the surfaces of monocytes which might be involved in nonspecific monocyte–T-cell interactions and not by inhibiting either monocyte Ia antigen expression or interleukin-1 production by monocytes.**

Previously, infections caused by nontuberculous mycobacteria were rare and mostly had been associated with chronic pulmonary infections in normal hosts. However, with the advent of acquired immunodeficiency syndrome, *Mycobacterium avium-Mycobacterium intracellulare* complex (MAC) has become the most common cause of disseminated infections in patients with acquired immunodeficiency syndrome. MAC infection is often accompanied by persistent bacteremia in patients with acquired immunodeficiency syndrome (16). Thus, a MAC infection develops as a form of opportunistic infection in the host in which T-cell dependent, macrophage-mediated immune responsiveness is depressed. In a preceding paper (37), we demonstrated that peripheral blood lymphocytes of patients with MAC infection indicated depressed in vitro lymphocyte transformation and interleukin-2 (IL-2) production after in vitro stimulation with purified protein derivative (PPD) as compared with the lymphocyte transformation and IL-2 production in patients with pulmonary tuberculosis or in tuberculin-positive healthy donors.

On the other hand, there have been several reports indicating that a variety of mycobacteria or their products induce suppression of in vitro cell-mediated immune response in human (6, 7, 14, 41) as well as in murine (4, 24, 28) systems. However, attempts to clarify the detailed mechanisms of suppression, cell natures involved, antigen specificity in interaction, and expression of cells have provided conflicting results, i.e., suppression was found in lymphocyte cultures only in donors infected or sensitized with a relevant mycobacterium or cross-reacting mycobacterium (20, 21, 24) or in donors regardless of whether their tuberculin skin test was positive or negative (6, 14, 18, 40, 41). Macrophages (15), T cells (7, 21, 24), both (18, 40, 41), or secreted soluble factors therefrom (34, 41) are responsible for suppression of in vitro cell-mediated immunity by mycobacteria or mycobacterial antigens.

The initiation of T-cell activation requires that T-cell receptors bind antigen in association with class II major histocompatibility, HLA-DR in humans, on antigen-presenting cells (APC) (17, 38). An absolute requirement for the presence of IL-1 in this induction of T-cell activation has been reported (22). In addition, there is evidence that cell surface molecules such as lymphocyte function-associated antigens (LFA) may facilitate early APC–T-cell interactions without antigen specificity and are required for subsequent T-cell activation (36).

In this study, we will demonstrate that monocytes and not T cells are the targets for the modulating effect of MAC in concanavalin A (ConA)- and PPD-induced T-cell proliferation and that neither the expression of HLA-DR nor IL-1 production by monocytes was influenced by the MAC organisms. On the other hand, expression of CD11b molecules as well as LeuM3 (CD14) molecules on monocytes was impaired by MAC and MAC-derived lipid. CD11b is one of the adhesion molecules and belongs to the LFA-1 family (32). An important role of these molecules on the surfaces of monocytes was illustrated by inhibiting ConA- and PPD-stimulated T-cell proliferation with the monoclonal antibody against these molecules. We postulate that the MAC organisms alter the membrane characteristics of monocytes and prevent the effective physical interaction required for activation of T cells. It is conceivable that as a survival

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mechanism, the MAC organisms could evade the immune system by influencing the regulation of adhesion molecule expression on the surface of antigen-presenting cells.

## MATERIALS AND METHODS

**Antigens and mitogens.** *M. avium* organisms (ATTC 15769, serovar 16) were provided by Kobayashi Pharmaceutical Co. Ltd., Osaka, Japan. They were treated at 60°C for 2 h before use. Stock suspensions of 10 mg/ml in physiological saline were sonicated and kept at −70°C and then diluted in RPMI 1640 medium for use. The lipid component was extracted in chloroform and methanol (2:1) as described by Bligh and Dyer (3). Finally, lipid extract from 2.0 g of dried *M. avium* was suspended in 10 ml of chloroform and methanol. This suspension was chosen as the original dilution and used in the culture after having been appropriately diluted in methanol. The amounts of endotoxin and lipopolysaccharide (LPS) in MAC and in MAC-derived lipid preparations were determined by the *Limulus* amebocyte lysate assay (*Limulus* ES-Test, Wako Chemicals, Osaka, Japan). The MAC solution was sonicated extensively before the assay. The amounts of endotoxin detected either in MAC organism suspension (500 μg/ml) or in MAC-derived lipid solution (200× dilution) were less than 6 pg/ml, the least amount detectable by this *Limulus* ES-Test kit.

ConA (Miles-Yeda Ltd., Rehovot, Israel) and phytohemagglutinin (PHA; Difco Laboratories, Detroit, Mich.) were employed to stimulate T cells nonspecifically. PPD of tuberculin from culture filtrates of *Mycobacterium tuberculosis* (PPD-S) was obtained from the Institute for Microbial Diseases, Osaka University, Osaka, Japan, and PPD from culture filtrates of *M. avium* (PPD-B) was donated by Kobayashi Pharmaceutical Co. Ltd.

**Mononuclear cell separation and preparation of T cells and monocytes.** Peripheral blood mononuclear cells (PBMC) taken from patient donors with tuberculosis and nontuberculous mycobacterial infections and from healthy donors whose tuberculin skin tests were both positive and negative were used. The cells were purified by density gradient centrifugation through a mixture of Ficoll-Hypaque as described previously (37). The resultant mononuclear cell suspension was separated into adherent and nonadherent cells as follows. A 5-ml sample of $2 \times 10^6$ PBMC per ml of RPMI 1640 medium supplemented with 10% fetal calf serum (M. A. Bioproducts, Walkersville, Md.) was placed in a plastic petri dish (60 × 15 mm; Kotai Kagaku Co. Ltd., Tokyo, Japan) and incubated at 37°C in 7.5% $CO_2$ in air for 2 h. Adherent cells were then extensively washed and were removed by adding 3 ml of cold EDTA solution and further incubating the dish at 4°C for 30 min. Adherent cells thus prepared were used as monocytes. In the experiments in which monocytes were added into T-cell proliferation, monocytes were previously irradiated (2,000 rads). T cells were obtained by treating nonadherent cells through a nylon wool column as described by Julius et al. (13). Monocytes and T cells thus prepared were examined for their cellular composition by fluorescence-activated cell sorter analysis. The monocyte population contained more than 95% $LeuM3^+$ cells. The T-cell population contained less than 0.5% $LeuM3^+$ and more than 85% $Leu4^+$ cells.

**Mitogen and antigen stimulation.** Cell-mediated immunity in vitro was assessed in PBMC as described elsewhere (37). PBMC were plated in triplicate at $5 \times 10^4$ cells per well in microplates (Falcon 3072; Becton Dickinson Labware, Oxnard, Calif.) in 200 μl of RPMI 1640 medium supplemented with 10% heat-inactivated pooled human serum and were cultured for 3 days with either 10 μg of ConA or 1 μg of PHA per ml and for 6 days with 10 μg of PPD-S or PPD-B per ml in humidified 7.5% $CO_2$ in air at 37°C. [$^3$H]thymidine (0.2 μCi) was added into each well 18 h before cells were harvested, and then [$^3$H]thymidine incorporation was evaluated.

To evaluate the effects of MAC and MAC-derived lipid on mitogen and PPD responses, either MAC organisms or MAC-derived lipid was added into the culture at the initiation of cultivation. In the culture with lipid, lipid extract in chloroform and methanol (2:1) was appropriately diluted in methanol, and 10 μl of diluted lipid was put into each well and dried in an air stream at 37°C before the addition of cells. In several experiments, the effects of MAC and MAC-derived lipid on PBMC responses were expressed in percent suppression calculated from the following formula:

$$\left\{100 - \frac{[\text{stimulator} + \text{MAC (cpm)}] - \text{MAC (cpm)}}{\text{stimulator (cpm)} - \text{cells alone (cpm)}}\right\} \times 100$$

In the experiment to determine the cell types responsible for the suppression, monocytes or purified T cells were precultured for 20 h with lipid. After harvesting, both lipid-treated and nontreated T cells were stimulated by ConA, PHA, or PPD in the presence of lipid-treated or nontreated monocytes.

IL-1β used to reconstitute T-cell responses was Genzyme's ultrapure human IL-1. Murine monoclonal antibodies employed for the suppression of T-cell responses included anti-LeuM3 (CD14) {RMO52 clone, immunoglobulin G2a(κ) [IgG2a(κ)]; Cosmo Bio Co., Ltd., Tokyo, Japan} and anti-CD18 (β-chain of LFA-1, BL5 clone, IgG1; Cosmo Bio Co., Ltd.). Murine myeloma protein, IgG1(κ), and IgG2a(κ) (Organon Teknika, West Chester, Pa.) were used as isotype controls of the monoclonal antibodies.

**Determination of cell surface antigen molecules.** Monocytes ($5 \times 10^5$ cells per ml) were incubated at 37°C in 7.5% $CO_2$ in air for 48 h in the presence and absence of MAC organisms or LPS from *Escherichia coli* strain 0111B4 (Difco Laboratories, Detroit, Mich.). After cultivation, extracellular MAC organisms were rinsed off, and the cells were washed three times with phosphate-buffered saline (pH 7.2) containing 1% bovine serum albumin and stained by treatment with the appropriate fluorescein- or phycoerythrin-labeled monoclonal antibodies which recognize the epitopes on the surfaces of cells. The cells were fixed in 1% formaldehyde and analyzed in a Facscan analyzer (Becton Dickinson). A total of $10^4$ cells, gated to exclude the cell debris and free MAC organisms that remained, were accumulated for each histogram. The data were plotted as the logarithm of fluorescence intensity against cell number. Cells displaying fluorescence intensities above the upper limit of the control distribution were considered positive. To determine the control distribution, fluorescein-conjugated mouse IgG2a was used in each analysis. Monocytes treated with either MAC organisms or MAC-derived lipid gave the same histogram patterns as nontreated monocytes did after staining with fluorescein-conjugated control mouse IgG. Labeled murine monoclonal antibodies employed included fluorescein-conjugated anti-LeuM3 (IgG2b) and phycoerythrin-conjugated anti-HLA-DR (IgG2a) and anti-CD11b (IgG2a). These conjugated antibodies and conjugated control mouse IgG were purchased from Becton Dickinson.

**Generation of IL-1-containing supernatants and assay for IL-1 activity.** Adherent cells ($5 \times 10^5$/ml) were cultured in the

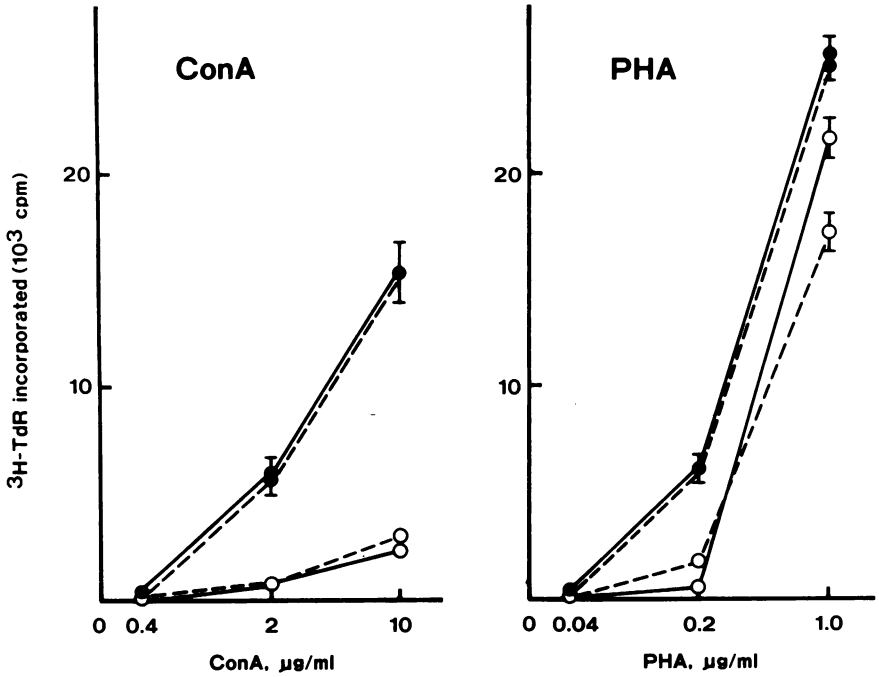



FIG. 1. Effect of MAC organisms and MAC-derived lipid on proliferative response of PBMC stimulated by ConA or PHA. Each point is the mean value of PBMC from three tuberculin skin test-positive healthy donors, and bars show standard errors. PBMC were cultured in vitro for 3 days. Symbols: ●——●, in medium; ●----●, in medium with methanol diluent; ○——○, in MAC (500 μg/ml); ○----○, in lipid (diluted 1:200). TdR, Thymidine.

presence of ConA (10 μg/ml) or MAC organisms (500 μg/ml) or both. After 24 h, the supernatants were collected, sterile filtered, and frozen at −20°C until used for IL-1 assays. The amount of IL-1 in the culture supernatants was determined by enzyme linked immunosorbent assay (ELISA), using a human IL-1β assay kit (Ohtsuka Assay Laboratory, Tokushima, Japan).

**Assay of cell viability.** At the time of harvest, the cells in the culture were suspended gently and diluted in eosin-Y solutions (final concentration, 0.2%), the total number was counted, and the percentage of viable cells determined. At least 200 cells were examined in each culture.

**Statistical analysis.** Experimental data were expressed as mean ± standard error of the mean. The significance of the difference between groups was calculated by Student's *t* test. Computer-assisted evaluation of the results was used to calculate the probability value in the data. A *P* value of 0.05 was used as the limit of statistical significance.

## RESULTS

**Effects of MAC organisms and MAC-derived lipid on mitogen-induced in vitro lymphocyte transformation.** The effects of MAC organisms and MAC-derived lipid on lymphocyte proliferation were first studied in a mitogen-driven system. PBMC ($5 \times 10^4$) were cultured together with either MAC organisms or MAC-derived lipid and stimulated by ConA and PHA. Untreated PBMC which were stimulated with either ConA or PHA showed a strong proliferative response after 3 days of incubation. Figure 1 shows the effects of MAC organisms and MAC-derived lipid on ConA and PHA responses. ConA response was significantly inhibited by MAC organisms (500 μg/ml) and lipid (200× dilution) at all concentrations of ConA examined, whereas a lesser inhibition was observed in PHA response. Methanol, a solvent for lipid, had no influence on ConA and PHA responses. To examine the possibility that MAC-induced suppression of the ConA response is due to the nonavailability of ConA after binding to MAC organisms, the ConA preparation (500 μg/ml) was first incubated at 37°C with MAC organisms (5 mg/ml) for 6 h. After centrifugation, the supernatant was assessed for its stimulatory activity in an in vitro lymphocyte proliferation assay. Mitogenic activity of ConA was not significantly decreased after incubation with MAC organisms, i.e., at the concentration of 10 μg of ConA per ml, proliferation of PBMC was $10.7 \times 10^3$ cpm in ConA without MAC treatment and $9.4 \times 10^3$ cpm in ConA incubated with MAC for 6 h at 37°C.

MAC alone did stimulate PBMC to proliferate from tuberculin-positive but not tuberculin-negative donors, although the extent of stimulation by MAC alone was small compared with that of ConA stimulation (less than $10^3$ cpm in MAC compared with $2 \times 10^4$ cpm in ConA). Coculture with MAC-derived lipid similarly inhibited ConA and, to a lesser degree, PHA responses of PBMC. The viability assessed by dye exclusion of MAC-treated and nontreated PBMC after 3 days of incubation was 85 and 89%, respectively.

Summarized data of MAC effects on the response of PBMC to ConA stimulation are shown in Fig. 2. The ability of MAC to suppress the response to ConA was similar among patients with tuberculosis, patients with nontuberculous mycobacterial infection, and healthy controls, irrespective of tuberculin reactivity of the donors.

**Effect of MAC organisms on PPD-induced in vitro lymphocyte transformation.** We next studied whether MAC could suppress the proliferative response of PBMC to optimal doses of PPD-S and PPD-B stimulation. The in vitro culture was carried out for 6 days. The addition of MAC to PBMC cultures with PPD (10 μg/ml) led to a reduction in the

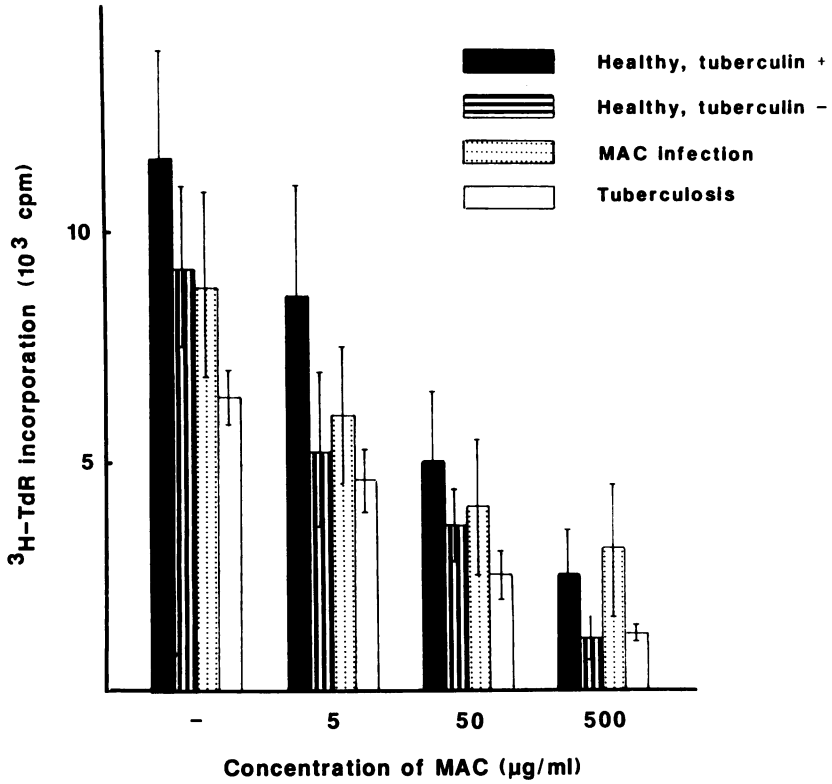



FIG. 2. Effect of MAC organisms on proliferative response of PBMC stimulated by ConA. PBMC were cultured in vitro for 3 days. Data are expressed as mean ± standard error. There were 4 and 5 healthy tuberculin test-positive and -negative donors, respectively, 7 donors with MAC infection, and 11 donors with tuberculosis. TdR, Thymidine; −, no MAC.

proliferative response observed with PPD alone, and the extent of suppression of the responses by MAC was in a dose-dependent manner (Fig. 3). The ability of MAC to suppress the PPD response was similar in patients with tuberculosis and tuberculin-positive healthy controls. MAC at concentrations of 5 and 50 μg/ml stimulated PBMC from tuberculin responders, the extent of which was about 1/5 of the stimulation by PPD-S (10 μg/ml), and at 500 μg of MAC per ml, the response was less than 1/10 of that with PPD-S stimulation. MAC at the high concentration of 500 μg/ml expressed no demonstrable effect on the survival of either PPD-stimulated or nonstimulated PBMC after 6 days of culture. The viability of cultured cells was as follows: 80% in control culture, 83% in PPD-S culture, 78% in MAC culture, and 85% in PPD-S plus MAC culture.

As described above, the extent of suppression by MAC organisms in ConA and PPD responses of PBMC was similar in patients and healthy individuals. The overall effects of MAC organisms expressed in percent suppression in all the

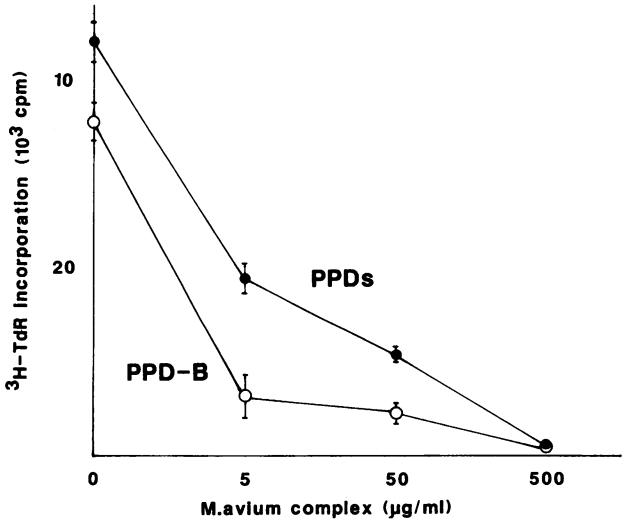



FIG. 3. Effect of MAC organisms on the proliferative response of PBMC stimulated by PPD. Each point is the mean value of PBMC from three tuberculin test-positive, healthy donors, and the bars show standard errors. PBMC were cultured for 6 days in the presence of PPD (10 μg/ml) and various doses of MAC organisms. PPDs was from *M. tuberculosis*, and PPD-B was from *M. intracellulare*. TdR, Thymidine.

TABLE 1. Effect on lymphocyte cultures of adding MAC organisms at different time intervals after stimulation with ConA

| Time (h) of MAC addition | [$^3$H]thymidine incorporation ($10^3$ cpm) | | | |
|---|---|---|---|---|
| | Expt. 1 | | Expt. 2 | |
| | Control (medium) | ConA[a] | Control (medium) | ConA[a] |
| No MAC | 0.2 | 24.7 | 0.2 | 22.1 |
| 0 | 1.6 | 1.5 (0) | 0.7 | 2.1 (7) |
| 3 | 2.2 | 9.5 (30) | 1.1 | 20.2 (86) |
| 6 | 2.3 | 11.3 (36) | 1.2 | 22.5 (95) |
| 24 | 0.6 | 22.1 (89) | 0.3 | 30.9 (139) |
| 48 | 0.2 | 25.8 (104) | 0.2 | 27.8 (126) |

[a] Percent activity in parentheses was determined by the equation in Materials and Methods.

TABLE 2. Effect of adding MAC-treated and nontreated monocytes on mitogen-induced T-cell proliferation

| Stimulator | [$^3$H]thymidine incorporation ($10^3$ cpm ± SE) in: | | | | | |
|---|---|---|---|---|---|---|
| | Medium | 5 × $10^4$ T cells plus: | | | | PBMC |
| | | 1 × $10^4$ monocytes | | 2 × $10^4$ monocytes | | |
| | | Nontreated | MAC treated | Nontreated | MAC treated | |
| Medium | 0.3 ± 0.0 | 0.3 ± 0.0 | 0.4 ± 0.0 | 0.2 ± 0.0 | 0.8 ± 0.0 | 0.2 ± 0.0 |
| ConA | 0.5 ± 0.0 | 8.9 ± 0.7 | 4.1 ± 0.5[a] | 17.6 ± 0.9 | 4.0 ± 0.4[a] | 24.3 ± 1.5 |
| PHA | 26.8 ± 0.9 | 23.2 ± 1.6 | 23.4 ± 3.6 | 28.1 ± 2.3 | 13.6 ± 1.5[b] | 37.7 ± 1.2 |

[a] Between nontreated and MAC-treated monocytes, $P < 0.01$.
[b] Between nontreated and MAC-treated monocytes, $0.01 < P < 0.05$.

donors examined (patients and healthy individuals combined) are 79.8% ± 3.0% in ConA responses ($n = 29$) 29.7% ± 3.6% in PHA responses ($n = 25$), and 72.5% ± 7.1% in PPD-S responses ($n = 18$).

**Effect of indomethacin on MAC-induced suppression.** To explore the possibility that the suppressive effect of MAC was due to prostaglandin release, indomethacin was added into the culture at concentrations of 0.1, 1.0, and 5.0 μg/ml together with either PPD or ConA. The suppressive effect of MAC on ConA responses and PPD responses was unaffected by the coculture of indomethacin (data not shown).

**Effect of addition of MAC organisms to PBMC cultures at different time intervals after stimulation with ConA.** In the next series of experiments, we tested at which step of culture the presence of MAC is required to inhibit T-cell proliferation in the presence of ConA. MAC organisms were added to PBMC cultures at the initiation of culture, that is, at the same time as ConA addition, and at 3, 6, 24, and 48 h after culture initiation. In all cases, [$^3$H]thymidine was added at 72 h after culture initiation. Maximum suppression was obtained when MAC was added at the start of culture (0 h), and the extent of suppression decreased as the time of addition was delayed (Table 1). No further significant suppression was observed when MAC was added at 24 h. A similar pattern of suppression was obtained in PPD responses (data not shown). These results indicate that MAC-induced suppressive activity evolves at an early stage in lymphocyte activation and that the suppression is not due to the toxicity of MAC.

**Involvement of adherent cells in MAC-mediated suppression.** We observed that MAC could suppress ConA, PPD, and, to a lesser degree, PHA responses and that the maximum suppressive effect was obtained when MAC organisms were added at the initiation of the culture. These results suggest that monocytes might be the cells influenced. Macrophages are required in the initial events in the immune response, phagocytosing, and processing and presenting antigenic information to T cells. Macrophages are also necessary in some mitogen-induced T-cell activation (30).

Therefore, we next examined the suppressive activity of MAC under conditions designed to alter macrophage–T-cell interaction. Adherent-cell depletion and cell-mixing experiments with T cells were carried out. At an optimum concentration of 10 μg of ConA per ml and at 1.0 μg of PHA per ml, ConA- and not PHA-driven T-cell proliferation systems were dependent on monocytes (Table 2). To test the concept that monocytes are the cells influenced in the MAC-mediated suppression of T-cell proliferation, we used a T-cell proliferative system in which only the monocytes were MAC treated. Monocyte monolayers were treated with MAC organisms for 2 h, after which the MAC organisms were rinsed off, the cells were washed three times with medium, and the monocytes were harvested. The viability of the MAC-treated monocytes was more than 85% and was comparable with that of nontreated monocytes. MAC-treated and nontreated monocytes were added to either ConA- or PHA-driven T-cell cultures. After 3 days of incubation, the proliferative responses were measured by [$^3$H]thymidine uptake. Incubation of T cells in the presence of nontreated monocytes stimulated with ConA led to a strong proliferative response. When monocytes had been previously treated

TABLE 3. Mitogen- and PPD-induced in vitro T-cell proliferation in the presence of monocytes: effect of pretreatment of monocytes and T cells with MAC-derived lipid[a]

| Cell type(s) | [$^3$H]thymidine incorporation ($10^3$ cpm ± SE) | | |
|---|---|---|---|
| | ConA | PHA | PPD |
| PBMC | 29.1 ± 1.1 | 34.1 ± 1.2 | 31.1 ± 0.8 |
| Nontreated T cells plus: | 0.4 ± 0.0 | 5.1 ± 0.4 | 0.1 ± 0.0 |
| Mϕ | 7.1 ± 0.8[b] | 17.2 ± 1.9[b] | 8.6 ± 0.7[b] |
| Mϕ + lipid-Mϕ | 6.6 ± 0.4 | 22.8 ± 0.8 | 21.2 ± 1.2 |
| Lipid-Mϕ | 0.5 ± 0.4[c] | 6.4 ± 0.8[c] | 0.6 ± 0.0[c] |
| Lipid-treated T cells plus: | 0.2 ± 0.0 | 4.1 ± 0.8 | 0.1 ± 0.0 |
| Mϕ | 5.5 ± 0.7[b] | 17.2 ± 0.9[b] | 11.8 ± 0.2[b] |
| Mϕ + lipid-Mϕ | 5.3 ± 0.6 | 21.0 ± 1.0 | 20.3 ± 1.4 |
| Lipid-Mϕ | 0.6 ± 0.1[c] | 5.1 ± 0.6[c] | 0.3 ± 0.1[c] |

[a] Purified T cells and monocytes (Mϕ) ($10^6$ cells per ml) were precultured with MAC-derived lipid (200× dilution) for 20 h, harvested, and washed extensively. T cells (5 × $10^4$/ml) and monocytes (1 × $10^4$/ml) were mixed and cultured together for 3 days in the presence of ConA or PHA and for 6 days in the presence of PPD. Control cultures without stimulation gave less than 200 cpm in both PBMC and T cells (nontreated and lipid treated).
[b,c] $P < 0.01$ between these values in each experiment.

TABLE 4. IL-1β activity in 24-h culture supernatants of monocytes

| Stimulator[a] | IL-1β activity (pg/ml) | | | |
|---|---|---|---|---|
| | Expt. 1 | Expt. 2 | Expt. 3 | Mean ± SEM |
| Medium | 510 | 280 | 580 | 457 ± 74 |
| ConA | 700 | 460 | 590 | 583 ± 57 |
| ConA + MAC | 1,150 | 730 | 920 | 933 ± 99[b] |
| MAC | 1,100 | 1,050 | 910 | 1,020 ± 46[b] |

[a] ConA, 10 μg/ml; MAC, 500 μg/ml.
[b] Compared with control, $0.01 < P < 0.05$.

with MAC organisms, the final proliferation of T cells was reduced. MAC-treated monocytes evoked a significant reduction in PHA response only when added at the higher percentage. PPD-driven T-cell responses were also inhibited by the addition of MAC-treated adherent cells into the culture (data not shown).

To further clarify the cell types and mechanisms responsible for suppression, we set up an experiment in which isolated monocytes and purified T cells were separately treated for 20 h with lipid before cultivation together and subsequent stimulation by ConA, PHA, and PPD. Monocytes and T cells were cultured with lipid for 20 h and washed extensively. Monocytes, lipid treated and nontreated, were mixed with T cells, lipid treated and nontreated, and cultured together with either ConA, PHA, or PPD-S. A representative result from three independent experiments is shown in Table 3. T-cell responses were suppressed by lipid-treated monocytes, unaffected by nontreated monocytes in the presence of ConA and PPD, and suppressed to a lesser extent by lipid-treated monocytes in the presence of PHA regardless of whether the T cells had been pretreated with lipid for 20 h. Eosin-Y exclusion revealed that the pretreatment of monocytes and T cells with MAC lipid for 20 h did not influence cell viability. Responses of lipid-pretreated T cells continued as long as nontreated monocytes coexisted in the culture. These results show that monocytes rather than T cells were the cells responsible for the MAC and MAC-derived lipid-mediated suppression of T-cell response.

**Effect of MAC on in vitro IL-1 production by monocytes.** T-cell proliferation requires both nominal antigen associated with major histocompatibility antigen class II molecules (Ia antigen) (17, 38) and de novo synthesis of IL-1 (22) by stimulated macrophages. We examined whether MAC-mediated immunosuppression was due to the impaired synthesis of IL-1 by monocytes. Monocytes produced IL-1β in their 24-h culture supernatants in both the presence and absence of ConA, and the presence of MAC organisms in the culture did not inhibit the IL-1β production (Table 4). To the contrary, MAC stimulated the adherent cells into production of substantial amounts of IL-1. The addition of exogenous IL-1β failed to restore T-cell suppression (Tables 5 and 6), thereby also confirming that reduced IL-1 production was not responsible for the MAC-induced suppression of the T-cell response. However, it is of note that IL-1 did suppress both ConA- and PPD-induced T-cell proliferation significantly. One unit of IL-1 used in the experiment in Tables 5 and 6 corresponds to 200 pg of IL-1β by enzyme-linked immunosorbent assay.

**Effect of MAC and MAC-derived lipid treatment on expression of monocyte surface antigens.** On the basis of the above observations, it is possible to assume that MAC suppressed T-cell proliferation by inhibiting Ia (HLA-DR) antigen expression on the surfaces of macrophages. The expression of I region-associated Ia antigen is essential for macrophages to function as APC during the induction of immune responses.

We next examined whether MAC and the MAC-derived lipid component could exert influence on Ia antigen expression on monocytes. Monocytes were cultured in vitro for 48 h in the presence and absence of MAC (500 μg/ml) or LPS (10 μg/ml). After being harvested and washed, cells were examined for surface antigens by a fluorescence antibody technique (Fig. 4). The expression of HLA-DR antigen was not inhibited at all by MAC. Neither the percentage of $Ia^+$ cells nor the density of the antigens on cells was altered by MAC treatment. On the other hand, a significant decrease in the density of antigens was observed in monocytes cultured with MAC organisms as detected by anti-LeuM3 (CD14) and anti-CR3 (CD11b) monoclonal antibodies. Expression of CD14 molecules was remarkably suppressed by MAC treatment, whereas LPS from *E. coli* had no influence on the expression of CD14. Expression of CR3 (CD11b) was also suppressed by MAC but not LPS treatment. An independent experiment with monocytes from three healthy donors showed the following data. Mean values of the percent $CD11b^+$ cells and mean fluorescence intensity are, respectively, 66.3% and 231 in nontreated monocytes, 62.2% and 104 in MAC-treated monocytes, and 63.6% and 232 in LPS-treated monocytes. Similar suppression of expression of CD14 and CD11b molecules was observed in monocytes cultured in the presence of MAC-derived lipids (data not shown).

**Effects of anti-CD18 and anti-LeuM3 (CD14) monoclonal antibodies on ConA, PHA, and PPD responses of PBMC.** As shown above, both MAC and MAC-derived lipid reduced the numbers of CD11b and LeuM3 molecules on monocytes. We examined whether these molecules might play any crucial role in T-cell proliferation by evaluating the effects of the addition of relevant monoclonal antibodies into the PBMC culture. Serial dilutions of monoclonal antibodies

TABLE 5. Effect on ConA-induced in vitro lymphocyte proliferation of adding IL-1β in the presence or absence of MAC organisms

| IL-1β (U/ml) | [$^3$H]thymidine incorporation ($10^3$ cpm ± SE) | | | | | |
|---|---|---|---|---|---|---|
| | Expt. 1 | | | Expt. 2 | | |
| | Medium | ConA | ConA + MAC | Medium | ConA | ConA + MAC |
| 0.0 | 0.5 ± 0.0 | 24.7 ± 1.6 | 6.5 ± 0.3 | 0.3 ± 0.0 | 11.6 ± 0.2 | 2.7 ± 0.1 |
| 0.5 | 1.0 ± 0.1 | 19.3 ± 0.7[a] | 7.9 ± 0.7 | 0.5 ± 0.1 | 8.8 ± 0.4[b] | 2.3 ± 0.3 |
| 2.5 | 0.6 ± 0.1 | 19.9 ± 1.1[a] | 7.0 ± 0.5 | 0.4 ± 0.1 | 8.3 ± 0.2[b] | 2.4 ± 0.2 |

[a] Compared with controls (no IL-1β), $0.05 < P$.
[b] Compared with controls, $P < 0.01$.

TABLE 6. Effect on PPD-induced in vitro lymphocyte proliferation of adding IL-1β in the presence or absence of MAC organisms

| IL-1β (U/ml) | [$^3$H]thymidine incorporation ($10^3$ cpm ± SE) | | | | | |
|---|---|---|---|---|---|---|
| | Expt. 1 | | | Expt. 2 | | |
| | Medium | PPD | PPD + MAC | Medium | PPD | PPD + MAC |
| 0.0 | 0.3 ± 0.0 | 38.5 ± 1.1 | 17.8 ± 2.1 | 0.4 ± 0.0 | 6.1 ± 2.4 | 0.8 ± 0.1 |
| 0.5 | 0.2 ± 0.0 | 30.6 ± 0.4[a] | 19.6 ± 1.2 | 0.3 ± 0.0 | 3.4 ± 0.3[b] | 0.4 ± 0.1 |
| 2.5 | 0.3 ± 0.1 | 32.0 ± 0.7[b] | 20.0 ± 2.8 | 0.4 ± 0.0 | 2.6 ± 0.2[a] | 1.6 ± 0.2 |

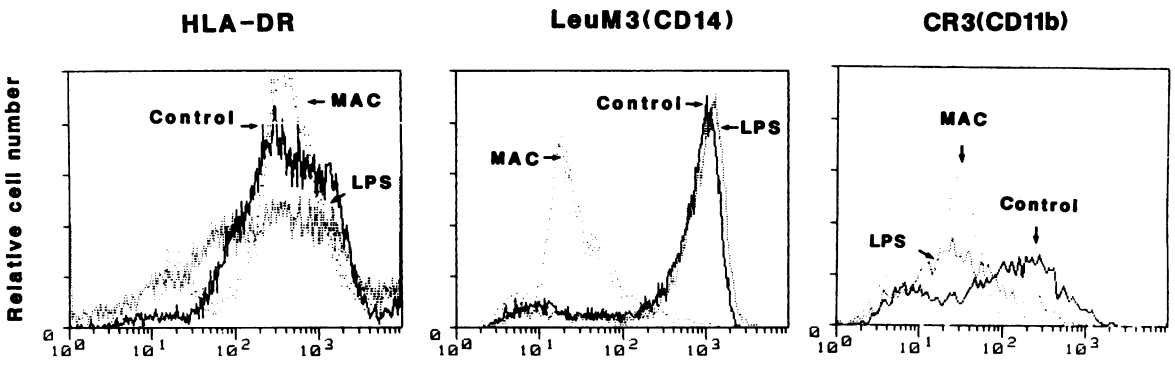
[a] Compared with controls (no IL-1β), $P < 0.01$.
[b] Compared with controls, $0.01 < P < 0.05$.

were added into the culture of PBMC and stimulated with adequate concentrations of ConA (10 μg/ml), PHA (1 μg/ml), or PPD (10 μg/ml). A representative result with anti-CD18 monoclonal antibody is shown in Fig. 5. The anti-CD18 monoclonal antibody (murine IgG1) defines the β-chain of the CD11b molecule which is a member of the LFA-1 family. Anti-CD18 antibody suppressed the ConA and PPD responses in a dose-dependent manner without significant suppression of PHA response. The mode of action of this monoclonal antibody on ConA, PHA, and PPD responses resembles the suppressive activity exerted by MAC organisms. The effect of anti-CD14 monoclonal antibody (murine IgG2a) on ConA- and PPD-induced T-cell proliferation varied in that the ConA- or PPD-stimulated T-cell response was suppressed in the lymphocytes of some donors but not in others. Mouse immunoglobulin isotypes (IgG1 and IgG2a) without antibody activity were included in the control cultures. They exerted no significant suppression at any of the dilutions examined.

## DISCUSSION

We have demonstrated that MAC organisms and MAC-derived lipid can induce in vitro suppression of the proliferative responses of PBMC not only in patients with *M. avium* infection and patients with tuberculosis but also in healthy individuals irrespective of a positive or negative tuberculin skin test. MAC significantly depressed ConA and PPD responses. However, the PHA response was only slightly affected by MAC. The findings that proliferative responses of PBMC depleted of adherent cells could no longer be suppressed by MAC indicated that monocytes were of crucial importance in this suppression. The suppression was not due to the toxicity of MAC, because the viability of PBMC after 6 days of culture with MAC organisms (500 μg/ml) was 78% and similar to the viability of PBMC cultured without MAC organisms. The fact that no further significant suppression was observed when MAC was added at 24 h (Table 1) also argues against the concern that suppression was attributed to the toxicity of MAC.

A wide variety of mycobacteria when added to normal mononuclear cell cultures inhibits their ability to undergo blastogenesis following mitogenic and antigenic stimulations. Ellner and Daniel (6) previously showed that arabinomannan removed from culture filtrates of *M. tuberculosis* suppressed the response of human lymphocytes to PPD and other antigens but not to PHA. Immunosuppression was




| | | | |
|---|---|---|---|
| Control | 962 * | 772 * | 295 * |
| MAC | 1,165 | 98 | 144 |
| LPS | 1,271 | 968 | 210 |

FIG. 4. Detection of HLA-DR, LeuM3 (CD14), and CR3 (CD11b) antigens on adherent cells after incubation for 48 h with MAC organisms. Adherent cells ($5 \times 10^5$) in medium supplemented with 10% pooled human serum were incubated in the presence and absence of MAC organisms (500 μg/ml) or LPS (10 μg/ml from *E. coli*). Cells were stained with phycoerythrin-conjugated anti-HLA-DR, fluorescein-conjugated anti-CD14, and phycoerythrin-conjugated anti-CD11b monoclonal antibody and analyzed in a Facscan analyzer. *, Mean fluorescence intensity.

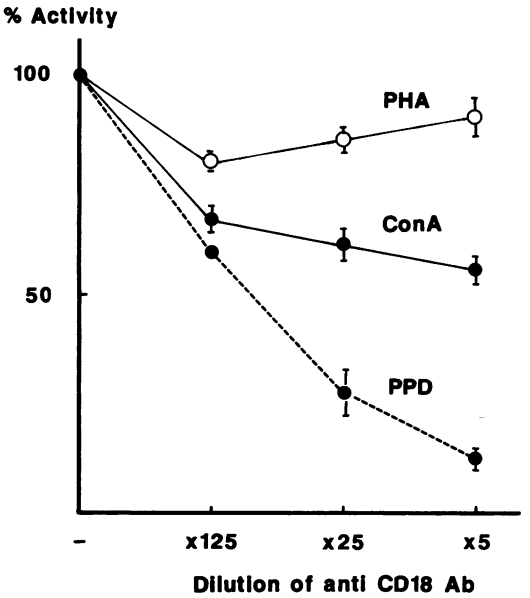



FIG. 5. Effect of anti-CD18 monoclonal antibody (Ab) on ConA-, PHA-, and PPD-induced proliferative responses of PBMC. PBMC from three tuberculin-positive, healthy donors were cultured for 3 days in vitro with ConA (10 μg/ml) or PHA (1.0 μg/ml) and for 6 days with PPD (10 μg/ml) in the presence of various doses of monoclonal antibody. Proliferative responses were assayed by measuring the [$^3$H]thymidine incorporated. Data are expressed as percent activity of the control culture with mouse IgG1 as the isotype control. Each point is the mean value of PBMC from three donors, and bars show standard errors. Anti-CD18 monoclonal antibody significantly suppressed ConA and PPD responses at all dilutions of antibody ($P < 0.01$). -, No anti-CD18 antibody.

observed in lymphocytes from both tuberculin skin test-negative and -positive donors. Mustafa and Godal (24) reported that in vitro activation with BCG of T cells from healthy individuals vaccinated with BCG led to the induction of suppressor cells that suppressed the proliferation of fresh T cells in response to a specific antigen. A certain number of studies were reported on the in vitro suppressive effects of *Mycobacterium leprae* and related antigens in patients with leprosy. Phenolic glycolipid of *M. leprae* induced general suppression of in vitro ConA responses unrelated to leprosy type (29). Experimental data support the role of *M. leprae*- and lepromin-induced suppressor macrophages and suppressor T cells in these patients in vitro, where they inhibit the mitogen and antigen responses of lymphocytes (25, 31). Recently, Fournie et al. (7) reported that phenolic glycolipids from mycobacteria inhibited in vitro human lymphoproliferative responses independent of the presence of APC or $CD8^+$ T cells. They concluded that the mechanism of inhibition appeared to be a nontoxic, functional effect on proliferating $CD4^+$ lymphocytes.

T-cell proliferation requires both nominal antigen associated with major histocompatibility class II molecules on APC (17, 38) and de novo synthesis of IL-1 (22) by stimulated macrophages. Studies of murine systems have indicated that Ia expression is increased on peritoneal macrophages treated with gamma-interferon and that this expression is suppressed by *Mycobacterium microti* in a dose-dependent fashion (15). Mshana et al. (23) reported that infection with live and not heat-killed *Mycobacterium kansasii* inhibited in vitro detection of Ia antigen which appeared on murine peritoneal macrophages after stimulation with gamma interferon. Our present study, however, indicated that MAC did not influence the expression of major histocompatibility class II antigen on the surface of monocytes. These divergent results may be attributed to the use of heat-killed MAC organisms in the present study. In addition, MAC did not suppress IL-1β production by monocytes. In the present study, MAC organisms rather produced IL-1β from monocytes. Addition of exertional IL-1β did suppress the ConA- and PPD-induced in vitro lymphocyte proliferation. IL-1β produced from monocytes by MAC stimulation does not, however, seem to play a major role in the MAC-induced suppression observed in our study. If MAC-induced suppression of ConA and PHA responses were solely due to the suppressive action of IL-1 produced by monocytes, the exertional IL-1 should further suppress the ConA plus MAC and PPD plus MAC responses. Tables 5 and 6 show that this is not the case.

A number of studies have reported immunosuppression by soluble factors obtained from mycobacterium-treated monocytes (41) including prostaglandin E2 (35). These suppressor factors will activate suppressor cells. In our study, we found that a role for suppressor factors is unlikely in MAC-induced suppression of T-cell proliferation for several reasons. First, MAC-induced suppressive activity was evident at an early stage in cultivation (Table 1). In experiment 2 of Table 1, the cultures in which MAC was added at 3 and 6 h no longer showed significant decreases in responses. Second, our cell mixing experiments (Table 3) show that MAC-derived lipid-treated monocytes exerted no suppressive activity in ConA-, PHA-, and PPD-stimulated lymphocyte proliferation where intact monocytes coexisted. Finally, the suppression by MAC was not blocked by indomethacin.

At present, the initiation of a specific T-cell response is believed to require not only activation through antigen-specific receptors on T cells but also antigen-independent interactions between accessory molecules. One such molecule is LFA-1 (36), which enhances the avidity of interaction between T cells and APC. C3bi receptor molecules (CR3) defined by anti-CD11b monoclonal antibody are within the family of LFA-1 (32). The molecules of LFA-1 are expressed on monocytes, T cells, and B cells. The ligand for LFA-1 is the intercellular adhesion molecule-1 (19) and is expressed on monocytes and T cells after activation (5). In the interaction between T cells and APC, binding of ligand to the LFA-1 molecule not only spatially orientates cells towards each other but also induces signals that regulate cell activation and differentiation (39). We showed in the present study that treatment of monocytes with either MAC or MAC-derived lipid resulted in a significant decrease in CD11b molecules on the cell surface. An important role of LFA-1 molecules was illustrated by inhibiting ConA- and PPD-stimulated T-cell proliferation with monoclonal antibody against CD18 (Fig. 5). Anti-CD18 monoclonal antibody defines the common β subunit of the LFA-1 molecules (32). The response of PHA-stimulated T-cells was, however, not inhibited by anti-CD18 monoclonal antibody. This is in agreement with the observation reported by Nishimura et al. (27) that in a murine system, the addition of anti-LFA-1 monoclonal antibody caused a significant inhibition of the T-cell response to ConA and not PHA stimulation. Blocking of macrophage–T-cell interaction by anti-LFA-1 antibody may be a major cause of the reduction of T-cell responses to ConA and PPD. The T-cell response to PHA, however, may evolve via an alternative pathway. This may explain why the PHA response depends less on accessory monocytes than ConA and PPD responses do. It may also explain why MAC

and MAC-derived lipid suppress T-cell responses in different manners between PHA and ConA stimulation. Furthermore, immunofluorescence demonstrated that the density of CD11b molecules was decreased on the surfaces of monocytes after MAC treatment. Recently, Blanchard et al. (2) demonstrated that an adhesion molecule, LFA-1, is involved in the lysis of mycobacterium-infected monocytes by IL-2-activated killer cells. Graham et al. (10) also demonstrated that CD11b/CD18(Mac-1) is involved in the phagocytosis of targets recognized by multiple receptors. Therefore, both MAC and MAC-derived lipid which have the ability to suppress the expression of the adhesion protein, as shown in the present study, serve to allow escape from killing by the host immune system.

It is of note that the densities of CD14 or LeuM3 molecules on monocytes were decreased after MAC treatment. As for the functional property of the CD14 (LeuM3) molecule in immune response, little evidence has been delineated. Analyses of complementary DNA and genomic clones of CD14 show that it has a novel structure and that it lies on chromosome 5 within a region containing other genes encoding growth factors and receptors (9). So far, no biological function has yet been ascribed to this molecule. Recently, Gidlund et al. (8) separated peripheral blood monocytes into LeuM3$^+$ and LeuM3$^-$ subsets. Their conclusion is that M3$^+$ monocytes mediate an antibody-dependent phagocytic activity, whereas M3$^-$ cells are more efficient in performing extracellular killing of the antibody-coated target cells. CD14 is a member of the family of phosphatidylinositol-anchored proteins which include LFA-3 (11). LFA-3 is another adhesion protein found on APC and binds to CD2 molecules on T cells (33). In the present study, MAC treatment substantially reduced CD14 expression on monocytes, although the effects of anti-CD14 monoclonal antibody on ConA- and PPD-induced T-cell responses were variable.

Of importance is the question of whether the MAC-induced in vitro suppression observed here actually occurs in vivo. Mycobacteria which are ingested into the phagocytes will interfere with the host immune response and can thus survive within the macrophages by escaping the actions of cytokines such as tumor necrosis factor (1), IL-2 (12), and gamma interferon (26), which are produced by activated T cells and which stimulate macrophages to kill bacteria within the cells. Lipoarabinomannan, one major mycobacterial surface component, has also been demonstrated to contribute directly to intracellular survival by inhibiting gamma interferon-mediated activation of macrophages (34). Several studies, including ours (37), demonstrated immunosuppression associated with infection by MAC organisms in both animals (4) and humans (37, 41). It is possible that the MAC infection is responsible for or contributes to the decreased mitogen and antigen responses found in patients infected with MAC, perhaps through the mechanisms analyzed in the present study.

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